Material & Methods

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# Results Section

# The Magnetic Pincher technique

## A short history

This project is a continuation of Valentin Laplaud's thesis work. During his PhD, Valentin created the Magnetic Pincher and made it into a fully functional technique. As his work focused on measurements of the cortex thickness & its fluctuations, my first task was to further develop the experimental set-up, protocols and software to go toward a mechanical characterization of the cortex.

I started out by learning the technique exactly as Valentin had been using it, but rapidly I had to transform it. Because I was now using adherent cells (3T3 fibroblasts) instead of immune cells, I developed a protocol to combine the Pincher with micropatterning. Then I rewrote the code responsible of 3D tracking of the beads, which could be improved thanks to the addition of a more precise piezo actuator to the microscope. Throughout my PhD I ended up rewriting most of the software, and redesigning a large part of the set-up, notably to integrate the nano-indentor. In this process, starting from a working version of the technique was of course immensely helpful. It gave me a standard to assess the correctness of my changes. It also allowed me to obtain results on live cells right from the beginning.

Yet this style of incremental development also came with a few challenges. For instance, we wanted to ensure a good backward compatibility of our data, so that our newest analysis code would still allow us to revisit data obtained by Valentin. But the most difficult aspect was conciliating the production of new experimental results with improvements of the technique itself. As we worked to address experimental problems or artifacts of the measure, it became complex to keep our results comparable. For instance the introduction of different bead coatings to prevent cells to phagocytose more beads during the experiment made the protocol much more robust. But it might also have affected slightly the local properties of the cell-bead contact area, in ways that we did not quantified. It is important to bear in mind this “instability” of the measurement tool, because it is consubstantial with this type of project.

During this work I also attempted to make the Magnetic Pincher available for other teams to use. It involved developing a simple magnetic field generating device (see *material & methods - Halbach array*) and writing analysis codes that others could easily appropriate (unfortunately the development of a full-fledged software with a user-friendly interface was not possible due to time-constraints, but I would have loved to try). We managed to port a version of the Magnetic Pincher to three other teams, and it lead to collaborative projects on cell migration, tissue mechanics or the role of membrane-to-cortex attachment[[1]](#footnote-1).

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| **BOX — History of the teams**  The PMMH laboratory — standing for *Physique et Mécanique des Milieux Hétérogènes* — is a very multidisciplinary lab which gathers teams interested in physical mechanics, active matter or complex media in a host of contexts and scales, from geophysics to biophysics. In this lab, the team supervised by Olivia du Roure and Julien Heuvingh have first been focusing on the mechanics of actin networks reconstituted in vitro. To study these gels the team developed many techniques based on magnetic colloids & their self-assembly properties [REF]. On the other hand, Matthieu Piel’s group in Institut Curie had developed a quantitative approach to cell biology, addressing questions such as cell volume control and the effect of mechanical stress on the nucleus [REF].  The collaboration between these teams was born with the aim of combining their research themes: use magnetic colloïds directly in living cells to measure the mechanical properties of organelles and link them to biological functions. Valentin Laplaud's work has demonstrated the relevance of this approach to the study of the actin cortex. |

## Principles

In essence, the Magnetic Pincher is a micro-scale rheometer. The idea is to apply a controlled force on a portion of a live cell and measure the resulting deformation. In practice, this is achieved by using a pair of superparamagnetic microbeads, one inside the cell, the other outside, and exposes them to an external, uniform magnetic field. As the beads attract each other, they end up positioned on either side of the cell surface — mostly including the membrane and the cortex. The externally applied magnetic field controls the magnetization of these two beads, and therefore the attractive force they apply on each other. Hence, by varying the field, we can make the beads indent more or less deep in the cell cortex. As it happens we acquire bright field microscopy films of the beads: with these images, we can track the beads positions corresponding to the set magnetic field. In summary the external magnetic field let us set the applied force, and the microscope allow us to monitor the cortex deformation as it is pinched by the beads: we have a cell cortex rheometer.

## Experimental set-up

From this image of a single cell cortex probed by two beads, let’s zoom out a little: the experimental chamber we place on the microscope contains thousands of cells, some with internalized beads, other without. As we add external beads and expose it all to a uniform magnetic field, all the beads simultaneously magnetize. Since there is no large scale gradient of field, the bead do not drift, but rather self-organize in pairs or chains aligned with the magnetic field lines. Sometimes, these pairs or chains form across a cell surface, and therefore constitute a Magnetic Pincher. Then an experiment consists in finding such occurrence, positioning the cell and pinching beads in the microscope field of view, and filming the beads while applying a set sequence of external magnetic field. Since the chamber contains a large number of cells and beads which are all magnetized at once, the number of suitable Magnetic Pinchers can easily be of several tens. By repeating this operation we can measure the cortices of 10 to 20 cells in each experimental chamber in approximately an hour and a half.

In this brief description of the experimental protocol, we have listed the main components of the experimental set-up: a microscope and a fast camera, the experimental chamber containing the cells, a device to generate the magnetic field. In addition, we use a piezoelectric actuator to control the objective vertical displacement with high precision, which allow us to acquire Z-scans of the beads; and of course a computer with a custom made software (written in Labview), controlling the other elements of the set-up in a synchronous manner through a Data Acquisition module (DAQ).

To generate a uniform magnetic field over our experimental chamber, several solutions exist. The most obvious one would be to use large permanent magnets, placed symmetrically on either side of the chamber. Another idea on the same line would be to use smaller magnets arranged in a circle around the sample to form a Halbach array (see Materials and Methods), which create a field with very little gradient. Both these solutions are easy to set up, but have the major drawback of being limited to magnetic fields of constant magnitude. This is why we opted for electromagnetic coils, placed in a quasi-Helmholtz configuration on either side of the experimental chamber. They are powered by a current of controlled intensity (set through the DAQ module), enabling us to program a sequence of magnetic field set points to be applied. The situation of our microscope stage looks as on **Fig. X**, and the complete set-up configuration is given in greater details in the *Material & Methods section*.

Overall, the most crucial element of this set-up is the superparamagnetic beads themselves. The validity and the precision of our measures entirely rely on the properties of these beads. In these experiments, we almost exclusively used M-450 Dynabeads, as they ally several key features:

* Very monodisperse in size; we can measure at the population level a standard deviation around the average diameter around 20 nm.
* Easy to track optically from bright field images, as below the beads forms a bright light spot, akin to a diffraction pattern. This light spot is easy to segment and tracking its center enables us to localize the bead with a subpixel resolution in the XY plane.
* Their size & density in magnetic elements make them relatively strong magnets: they can exerce forces from a few pN up to 1 nN, which is very relevant for a material such as the cortex.

All these properties are described extensively in the *Material & Methods section*.

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| **BOX — The choice of 3T3 Fibroblast as our model cell line**  As often in biology, the choice of our model cell line has been partially fortuitous. The Magnetic Pincher had been used before on amoeboid-like cells such as mice dendritic cells, or *Dictyostelium discoideum*. However the collaboration with the group of Alba Diz-Muñoz on Membrane-to-cortex attachment led us to adapt the technique to the cell type they were using, NIH 3T3 fibroblasts. After this collaboration, we chose to keep using the cell line — we ordered a fresh one from the ATCC cell bank — as I had already developed a set of protocols dedicated to it.  Yet, NIH 3T3 Fibroblasts are a relevant choice for this project for several reasons:   * Easy to culture and at the lowest level of the biosafety scale, they are a good candidate to be cultured and experimented with in a Physics lab which do not have the same level of equipment as a Biology one. * They have been a popular model cell line for quite a long time, thus literature describing them and their properties is quite abundant. * They are good candidate for viral transductions, and so we found that many useful cell biology tools such as actin labels or optogenetic systems where often available in 3T3s. * As mesenchymal cells, they differ completely from the amoeboid cells used on so far, making this project a good complement to previous work. |

## Preparation

The first condition for the Magnetic Pincher to work is to make sure beads can be internalized by cells. Indeed it is not obvious that cells would be able to spontaneously ingest large non-biological objects such as our 4.5 µm beads. This is the reason why the technique was first developed in immune cells (primary mice dendritic cells) or Dictyostelium, which are known for their phagocytic or macropinocytic activity.

In this work we chose from the beginning to work with 3T3 fibroblasts cells (see BOX) and the protocol had to be adapted in several ways. First we had to make sure these "non-professional phagocytic" cells[[2]](#footnote-2) would properly ingest beads. We tried to incubate them with cells in the conditions of culture, but the number of bead internalized stayed exceedingly low. We devised a protocol to coat the beads with fibronectin, which drastically increased the rate of ingestion. As a result, the preparation of each Magnetic Pincher experiment started with the addition of fibronectin coated Dynabeads in the cell culture flasks, two days before the experiment.

Second, as adherent cells, 3T3s die if they are not allowed to adhere to a substrate; but when they can, they spread largely and become very flat. When they have this shape, it is difficult to use the Magnetic Pincher, as the inner bead is larger than the typical height of a spread fibroblast. It is pushed toward the center of the cell where it acts as a tent pole. Because of the curvature of the cell surface, it is very hard in these conditions to form a pair with another bead outside; and if it happens, the beads are usually in different planes, thus impossible to image. This is why we used fibronectin micropatterns, so as limiting their spreading while allowing them to adhere. In practice, we used homemade glass bottom petri dishes, where the glass had been passivated with PLL-Peg, and patterned with fibronectin discs. Since the average diameter of 3T3 cells in suspension is roughly 18 µm, they form a quasi-hemispheric shape when adhering on discs that have a diameter close to this value. Thus, we picked 20 µm as our standard pattern size. Because the quality of these micropatterned substrates tends to degrade with time, we manufactured them less than a week before the experiment, and performed the last step of fibronectin addition only the day before.

An experiment with the Magnetic Pincher is typically performed over a whole day. In the morning, cells are detached with trypsin and transferred in the experimental chambers. After 20 minutes, cells start adhering to the fibronectin discs. The chambers are “flushed” using two pipets — one inlet, one outlet, to create a flow — which removes the non-adhering cells. The chambers are then placed in the incubator for at least 2h, so the cells can reach a relative steady state of adhesion [ref for adhesion process ?]. Before bringing a chamber to the microscope, beads were added to the medium. These are supposed to be the outer beads, complementing those that have been ingested by the cells. Therefore we devised this time a way to prevent cells to ingest these beads: Dynabeads functionalized with streptavidin were grafted with mPeg-biotin, effectively covering them with a slippery brush of Peg chains. The resulting beads are non-adhesive to the cells, and we never observed an ingestion event.

## Performing the measure

The chamber is placed on the microscope stage, the electromagnetic coils — not powered for now — are placed on either sides. We use simple bright field microscopy to image cells and beads, as the tracking method relies on the bright spot forming below the beads. However we use a 100X objective (NA = 1.4) and a fast camera with 16-bit depth to increase the precision of the beads’ center localization.

For each chamber, we start by acquiring Z-stacks of single beads using a piezo actuator to move the objective vertically. These scans are made for 5 to 10 beads, and will be used to generate a typical profile in depth of the bead. We call such profiles “Depthograph” in analogy with kymographs. They will be used later for image analysis, to compute the relative positions of the beads along the z-axis.

We switch on the magnetic field, and using a Labview software to control the different elements of the set-up, we start taking films of cells while simultaneously setting the applied field. There are mostly two ways we use the Magnetic Pincher: first, with a low & constant field, the system behave as a caliper. Since the beads do not indent the cortex much, tracking their positions gives a measure of the cortical thickness and its fluctuations in time. In practice we used 5 mT as a standard magnetic field. We acquired images by tacking short Z-stacks every 600 ms; each stack comprised 3 frames distant of 0.5 µm along the z-axis. These triplets enabled us to reach a better precision in the 3D-tracking of the beads. These acquisitions lasted typically 5 to 10 minutes.

The second approach consists in imposing a series of force ramps to compress the cortex while monitoring the beads movement. From these films, we compute the cortex elasticity. After several iterations, we decided to use the following sequence [Fig. X]. It starts and finishes with an acquisition identical to the one described earlier: the field fixed at 5 mT, an image triplet taken each 600 ms, with a z-step of 0.5 µm, for 6 seconds. Next the field is smoothly decreased and maintained for 2 seconds at a low value, typically 2 mT. The point is to release the force before the compression, in order to better estimate the non-deformed thickness of the cortex. Then the compression itself takes place: the field is increased from this low value up to the maximum than the coils can generate (typically 55 mT). As this occurs rapidly over 1.5 seconds, the image acquisition frequency is increased to roughly 80 Hz. This is crucial to obtain a reliable force-indentation curve, with enough points to fit a mechanical model. The compression is followed by a relaxation to the resting value of 5 mT, and finally a series of triplets is acquired for another 6 seconds.

Overall this whole sequence is about 20 seconds long and is repeated 5 to 10 times. The time-scales are important here: as the cortex is a very dynamic object with a typical turnover time of 10 to 20 seconds, the compression has to be much faster to correspond to a sort of “snapshot” of the cortex mechanical state. On the other hand, the delay of 20 seconds between successive compressions is set to let the turnover happen. Indeed, it is important to have in mind that when we perform this routine, it affects simultaneously all the beads present in the chamber. Thus as we observed one cell being compressed, all the other suitable cells undergo the same compressions. However we never observed a long-term effect on the mechanical properties of cells measured over a long duration in a single chamber [FIG TO PROVE]. Our working hypothesis on this is that the 20 seconds delay between compressions is probably enough to let the cortex reorganize, under a lower force. However we have not systematically investigated the effect of this parameter on a potential long-term load-adaptation of the cortex.

## Image analysis

In order to get the cortex thickness from these bright field images, we developed a 3D-tracking algorithm to compute the trajectories of the beads pinching the cortex. The thickness is deduced from the 3D center-to-center distance of the two beads by simply subtracting the beads diameter. We start by tracking the beads in the XY-plane: first, a threshold-based segmentation of all the beads’ bright spot in a film (using Fiji’s “Analyze Particles” function). Then we perform a simple matching operation to establish the trajectories of the two beads of interest. Beads segmented in frame i are matched with those of frame i+1 by finding the solution that minimize the total squared displacement (using an implementation of the Hungarian algorithm in Python).

These trajectories are only 2D projections of the real 3D movement of the beads, as we have yet to compute the position of the beads along the z-axis. To do so, we use the above-mentioned depthograph: a typical YZ-profile of the beads, which we can use as a kind of vertical scale.

A depthograph is made for each experimental chamber using Z-stacks of single beads acquired in that chamber. The Z-stacks consist in 401 frames, vertically distant by 20 nm, spanning from the bead equatorial plane to 8 µm below — like a vertical scan of the optical patterns forming below the bead in bright field. We generate a vertical section of the bead image, passing through its center along the y-axis — in other words a kymograph, with depth instead of time. The resulting image is a YZ-section of the bead, where each row is a profile of the bead along the y-axis.

The vertical tracking of a bead then consists in finding where its profile fits the best in the depthograph. We take a line passing through the bead center along the y-axis, and compare the pixel intensity profile to every row of the depthograph. To find the best match, we use a cost function that is the integral of squared difference of two pixel intensity profiles. The row of the depthograph that minimizes this cost function is the most similar to the profile of the tracked bead.

For both beads pinching the cortex, we find to which row of the depthograph they match best. From their relative position on this common scale, we can compute the relative vertical distance between our beads — as each row in the depthograph correspond to a 20 nm step.

This process can be improved by using images n-tuples. As mentioned before, when the experiment does not require very fast imaging, we usually acquire for each time-point a short Z-stack of 3 frames distant of 0.5 µm. Thus, 3 profiles can be matched at once with the depthograph, allowing us to get a more precise tracking of the bead in the vertical direction.

Overall, this operation gives us a vertical distance and together with the tracking in the XY-plane, it allows us to compute the 3D center-to-center distance between the beads pinching the cortex. Knowing the bead typical diameter, we simply determine the cortex thickness. We estimated the precision of the tracking to be around 10 nm in 2D, and 50 nm in 3D. The details of the algorithm, as well as the computation of the precision are given in the *Material and Methods section*.

## Force computation

When the relative positions of the beads of interest are known for all time points, the attractive force they exert on each other can be calculated. So far we have used a shortcut by claiming that fixing the external magnetic field allows us to control this force. In reality, it also depends of the thickness of the cortex and the orientation of the pair of beads, which behave like two magnetic dipoles:

Where µ0 is the vacuum magnetic permeability, m is the magnetic moment of the beads (considered identical here), d is the 3D center-to-center distance and α is the angle between the direction of the magnetic field and the line joining the two beads’ centers. The magnetic moment m is computed knowing the beads volume V and the magnetization M as a function of the external field B (see Materials and Methods):

Note that for the beads of interest, varies actually quite little, since with the bead diameter, while the cortex thickness h stays mostly between 100 and 500 nm. On the other hand, m varies almost linearly with B for low fields. This is why we can say that setting the external magnetic field allows us to *mostly* control the attractive force magnitude.

Finally, our algorithm also includes corrective terms to take into account the presence of other beads immediately around the two pinching the cortex. These neighbors can indeed locally increase the magnetic field, and contribute to the pinching force with an additive term. The calculation of these corrections is detailed in Materials and Methods.

## Mechanical models

Modelling the actin cortex in a mechanistically accurate way is a tremendously complex task. The cortex is viscoelastic, highly dynamic, probably anisotropic, and surely composed of many other elements beside actin. Just like cell compressions with AFM had to be first analyzed with the very classic Hertz contact model, we chose to start with a set of simple hypotheses. We consider the cortex to be a thin layer, linearly elastic and isotropic, indented symmetrically by two large spheres with no contact adhesion. To account for this geometry, a model has been derived in 2002 by Chadwick [Ref]. It results in a force-indentation relation expressed as such:

This result can be understood as a variation of the usual Hertz contact formula, where; in this case, a sphere indent a semi-infinite medium and the deformed region grows radially and in the depth of the material. Here, the layer is very thin compared to the spheres radius, and the deformed region grows only radially as the indentation increase. Chadwick’s formula can be easily re-*demonstrated in scaling laws.* DEMONSTRATION HERE.

This demonstration highlights the assumptions under which this formula is valid:

The first condition implies small deformations; the second means the layer

SHOW EXEMPLES OF FITS AND DISCUSS.

## Comments on the method

**Cell geometry.** Using 20 µm discs of fibronectins as our cells substrate is a way to constraint their geometry. They mostly adopt rounded shape, with a region where their cortex is almost vertical. This lets the beads position themselves in the same plane, on either side of the cell surface. However we can’t exclude that we are probing a specific region of the cortex, close to the contact with the substrate, which might be richer in actin.

**Beads internalization.** We often performed experiments with cells expressing LifeAct-EGFP. We never observed a defined actin cage remaining around the internalized beads. In an experiment performed on macrophages (see HoxB8 cells, see materiels & methods), we observed the thickness of the layer between two beads as the outer one is being internalized. On this example (Fig. X) we measured a remaining thickness of zero just a few seconds after the internalization. This points toward the fact that the endocytic machinery is highly dynamic and disassemble very quickly after bead internalization.

**Strain rate dependency**. Our set-up can perform indentations at various speeds. We could vary the shear rate over slightly more than a decade. On the one hand, the minimum frequency is limited by the cortex turn-over rate, which makes the interpretation of very slow indentations difficult. On the other hand, the maximum speed of our camera caps the frequencies we can reach. \*\*\* show and interpret results. \*\*\* Present our typical shear rate.

**Cell mechanical identity**. The intra-cell variability is much lower than the inter-cell one. Despite the constant turnover the cortex of a cell seems to maintain a sort of mechanical identity.

**Mechanical memory of the actin cortex**. Comment on the fact that we do not observe a systematic effect of repeated compressions over a long time.

**The cortex behaves as a solid even at very long timescales**. Comment on the small increase in thickness before compressions, which let us believe that the cortex never fully adapt to the load imposed by the beads under 5 mT.

## Non-linear models

# Materials & Methods

# Cell Culture

## 3T3 ATCC-2023 fibroblasts

These cells from the ATCC cell bank were purchased from LGC standards (#ATCC-CRL-1658) in January 2023. They were amplified and frozen at low passage numbers. They were also used as a base for the creation of a stable line expressing LifeAct-EGFP (see below).

They were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with GlutaMAX (#61965026, Thermofischer) supplemented with fetal bovine serum (FBS — 10% of final volume, #S1810-500, Biowest, France) and penicillin-streptomycin (PS — 1% of final volume, #15070063, Thermo Fisher, USA). They were incubated at 37°C in 5% CO2 atmosphere. They were passed every 2 - 3 days with 1/4 to 1/10 dilution, using TrypLE (#12605036, Thermo Fisher, USA). We ensured that they do not exceed 80% confluence.

## 3T3 αSFL fibroblasts

These cells were given by Sergio Lembo and Alba Diz-Muñoz as part of a collaborative project. The development process of these cell lines was fully done by S. L., and is detailed in [REF]. In our lab, we conserved frozen vials and cultured these cells using our culture medium, where the FBS had been replaced by the one supplied by S. L.

Briefly, these cells are 3T3 fibroblasts (also #ATCC-CRL-1658) engineered to express a synthetic membrane-to-cortex linker upon induction with overnight doxycycline treatment (16h with 1 μg/mL doxycylin #446060050, Thermo Fisher, USA **CHECK IT IN JUSTORDERIT !!).** Cell lines with diverse level of expression of the linker were obtained by subcloning.

We mostly worked with cell lines expressing the linker called “iMC”, composed of 3 functional domains: an actin binding domain (Utrophin), a mCherry reporter and a plasma membrane binding domain (Lyn motif). These domain were linked by flexible coil-coiled designed so that the total protein length would be about 10 nm. This linker was selected among many other combinations “Actin binding domain + plasma membrane binding domain” with an agar pad assay: upon expression of the iMC-linker, cells pressed with an agar pad exhibited significantly less blebbing than control cells without the linker.

Another type of linker, the “iMC-6FP”, included in its central region 5 non-fluorescent mCherry molecules in addition to the other domains of the iMC-linker. This resulted in a protein around 30 nm long. It was also expressed by 3T3 fibroblasts upon induction by doxycycline.

## HOX B8 macrophages

These cells were developed by Perrine Verdys under the supervision of Renaud Poincloux. All details regarding their development and culture can be found in (1,2). The cells mentioned in this work were cultured by P. V. and brought by R. P. for experiments over a week, using the medium and reagents they supplied.

In short, myeloid progenitors were extracted from mouse bone marrow and immortalized through retroviral transduction, enabling conditional expression of the HoxB8 homeobox gene. HoxB8 progenitors could be conserved and cultured in the long term, and differentiated in macrophages when needed upon exposition to mouse macrophage colony-stimulating factor (mM-CSF).

HoxB8 progenitors were knocked-out for ezrin, radixin and moesin using CRISPR Cas9. The absence of expression for the three proteins was verified with flow cytometry and immunoblotting. Triple knock out macrophages were differentiated from these progenitors.

## Cell lines conservation

3T3 cells (αSFL and ATCC-2023) were systematically amplified and frozen at low passage numbers. Cells were frozen in 1mL aliquots of roughly 2 millions cells/mL. To do so cells were detached using TrypLE (12605036, Thermo Fisher, USA), centrifuged in culture medium and the pellet was suspended in a mix of fetal calf serum (50%), and culture medium (45%) and DMSO (5%). They were conserved at -150°C and thawed when needed. The total passage numbers was always kept below 20, and rarely went above 10 throughout this work.

# LifeAct-EGFP Transduction

A stable cell line of 3T3 ATCC-2023 fibroblasts expressing LifeAct-EGFP was produced by lentiviral transduction followed by FACS sorting.

To do so, I prepared a mix of 200 µL of OptiMEM (Thermofischer), 4 µL of Lipofectamin 2000 (Thermofischer) and 2 µg of DNA, which include:

- 0.3 µg of pMD2.G — envelope protein (gift from Francois-Xavier Gobert, Institut Curie)

- 0.8 µg of PsPax2 — reverse transcriptase, capside, integrase (gift from Francois-Xavier Gobert, Institut Curie)

- 0.9 µg of the plasmid of interest — pLenti Lifeact-EGFP BlastR (Addgene Plasmid #84383)

I plated 1.6 million HEK-293FT cells (gift from the lab of Nicolas Manel) in 2mL of DMEM (ref 61965059, Thermofischer) and supplemented with the mix. 4 hours later I replaced the medium with 3mL of the 3T3 culture medium.

The next day I harvested the viruses by aspiring the medium with a syringe and filtered it with a 0.45 µm membrane. I added 1 mL of virus solution in three wells of a 6-wells plate containing cultures of 3T3 ATCC-2023 fibroblasts at different concentrations (around 10, 25 and 50% confluency).

The third day I washed the 3T3 cells twice with PBS and renewed the medium. On the fourth day I assessed in which of the three wells the cells had the most fluorescence signal while retaining a normal phenotype. I amplified these cells and proceeded to a FACS sorting to keep only cells within a narrow window of LifeAct-EGFP expression. These cells were amplified and frozen. The resulting stable cell line was called 3T3 ATCC LaGFP 02-01.

# Micro-patterned Chambers

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| *# This could be placed here or before, in the presentation of the technique in the results section.*  3T3 fibroblasts are adhering cells that spread a lot when cultured on a 2D substrate. When they have this shape, it is difficult to use the Magnetic Pincher because of what we nicknamed the “tent-pole effect”: the inner bead is larger than the typical height of a spread fibroblast. The bead will be brought toward the center of the cell where it acts as a “tent-pole”. Because of the curvature of the cell surface, it is very hard in these conditions to form a pair with another bead outside; and if it happens, the beads are usually in different planes, thus impossible to image.  On the other hand, 3T3 cells do not survive for a very long time when they cannot adhere to a substrate. This is why we used fibronectin micropatterns to limit their spreading without preventing them from adhering. More precisely, we developed a protocol to make fluorodish-like chambers, where the glass bottom is passivated with PLL-Peg, and micropatterned with fibronectin discs.  Since the average diameter of 3T3 cells in suspension is roughly 18 µm, they form a quasi-hemispheric shape when adhering on discs that have a diameter close to this value. Thus, we picked 20 µm as our standard pattern size.  *# It’d be nice to have a figure to illustrate the situation of beads in/out the cell on different patterns* |

This protocol was largely adapted from the method detailed in (3,4).

**Photomask design.** We designed the masks geometry with the software CleWin5. The masks were purchased from JD-Photodata (UK). We used masks made of quartz, because glass absorbs the deep-UV used for the patterning.

**Petri dish preparation.** We use 35 mm petri dishes with a 20 mm hole cut in the plastic bottom. Such dishes can either be bought or fabricated. We used a laser cutter (Epilog Laser, USA), which can cut dishes by batches with good precision on the centering.

**Coverslip passivation with PLL-Peg**. Round glass coverslips (diameter 25 mm, thickness #1) are cleaned with ethanol and exposed to plasma for 2 minutes. Then they are placed over 50 µL droplets of a 0.1 mg/mL PLL-Peg solution [PLL(20)-g[3.5]-PEG(2) from SuSoS, Switerland, diluted in HEPES 10 mM pH 7.4, and filtered with a 0.45 µm membrane]; the coverslips are incubated with the solution for 40 minutes. They are then removed, rinsed with milliQ water and gently dried with a tissue (without contact with the coated surface).

**Micropatterning with deep-UV.** The mask was cleaned with isopropanol, then the metallic side was exposed to deep-UV for 10 minutes. We used a UV lamp with wavelength λ = 254 nm and power P = 7 mW/m² (UVO Cleaner, Jelight) placed under a fume hood because of ozone emissions. Then, for each coated coverslip, a drop of 11 µL of milliQ water was placed on the metallic surface of the mask. Coverslips were gently placed on top of these droplets, coated side down, so as not to retain any air bubble between the glass and the mask surface. Then remove the excess of water by blowing compressed air from the top: each coverslip should be stuck to the mask by a very thin water film. The mask was exposed again to deep-UV-light for 10 minutes, this time with the non-metallic face toward the UV source (so that the light properly go through the mask before reaching the coverslips). Finally, the mask was covered with a puddle of milliQ water to allow the coverslips to detach. They were removed and dried.

**Experimental chamber manufacturing.** We used non-toxic silicon glue (Silicone SA 500, Zolux, France), to attach the patterned coverslip to the cut petri dish. Using a syringe, the glue was applied in a thin line around the hole, on the bottom side of the dish. A PLL-Peg patterned coverslip is then gently pressed onto the circle of glue. The glue was left to dry for at least 4 hours.

**Fibronectin addition.** We used either a solution of fibronectin (#F1141, Sigma-Aldrich) [10 µg/mL fibronectin in NaHCO3 buffer pH 8.3, filtered with a 0.2 µm membrane] or a mix of fibronectin and Alexa Fluor™-conjugated fibrinogen (typically Alexa Fluor™ 647, #F35200, ThermoFischer) [same recipe as before plus 4µg/mL fibrinogen], which allow a precise visualization of the patterns. Patterned dishes were incubated for 30 minutes with 125 µL of solution (using parafilm discs to spread the solution on the glass surface). The dishes were then rinsed twice with PBS.

# Magnetic Beads properties

## Choice of the magnetic bead type

The properties of the superparamagnetic beads are crucial to the feasibility and the precision of the Magnetic Pincher technique. The size of the beads is particularly important: they need to be large enough to generate a sufficient dipolar force and for their position to be accurately determined with bright field microscopy. We have identified the Dynabeads M-450 Epoxy (#14011 Dynal, Thermo Fisher, USA) as the best choice since they ally many important features:

1. Beads from the Dynabead line are very monodisperse in diameter. A given batch of M-450 Dynabeads typically has an average diameter close to 4.5 µm with a standard deviation of less than 25 nm. This is crucial because as discussed below, uncertainty about bead size directly affects our absolute measurement error. Because the average diameter varies from batch to batch, it is systematically re-measured when we order a new one.
2. M-450 possess one of the highest density of magnetic particles (5) and the largest size of all Dynabeads. Thus they can acquire high magnetic moment magnitudes, which result in a large range of pinching forces: from 1 pN to more than 1 nN. Moreover, like other Dynabeads, they have a very small residual magnetization, which ensures that they stop attracting when the field is brought back to zero.
3. Optical properties: when observed under transmitted bright light illumination at high magnification, an intense light spot forms below the bead center (Fig. X). By computing the center of mass of this light spot, using the light intensity (pixel value) as a weight we can localize the center of the beads with a resolution that overcomes the diffraction limit.
4. They are relatively easy to take up for cells. This depend on the cell type and can be tuned by coating the beads with diverse molecules, but every cell type considered for this experiment so far have proved able to take up M-450 beads.

This technique have also been performed successfully using M-270 beads (#14301 Dynal, Thermo Fisher, USA), which are smaller (typical diameter: 2.7 µm) and magnetize less strongly. The method did not work when attempted with MyOne™ beads (typical diameter: 1 µm). The M-450 beads are nonetheless the best choice in terms of robustness and precision.

## Tracking in 3D

When observed under transmitted bright light illumination at high magnification, an intense light spot forms below the bead center (Fig. 5C). Computing the center of mass of this light spot, using the light intensity (pixel value) as a weight allows the center of the beads to be localized with a resolution that overcomes the diffraction limit: the precision of their localization in the XY place is 2 nm (6,7). In practice, this detection of the center in 2D is done with a very simple ImageJ routine (see below).

However, beads pinching a cell are not always at the same altitude: the distance along the z-axis often has a significant contribution. It is then necessary to detect the relative positions of the beads in 3D, which is a little bit trickier. To do so, with standard image analysis techniques (no Machine Learning yet!) our method is based on the generation of a “depthograph” — a typical YZ-profile of the light patterns formed below the beads, just like a kymograph in depth. It serves as a common reference: if for an image of beads, we can find for each beads where they are in the depthograph, we know their relative positions along the z-axis.

To make depthographs, we take Z-stacks of beads (401 frames with a 20 nm step, from the equatorial plane downward). Then on each frame we draw a vertical line passing by the center of the bead, and we take the pixel intensity profile along this line. This gives us a 2-dimensional map of the bead depth in the YZ-plane. We repeat this operation for several beads and average them together to obtain our depthograph.

Then, to localize beads in a frame of a time-lapse, we detect their centers in XY using the method described above. For each bead, we proceed as before, drawing a vertical line passing through the center of the bead, and taking the pixel intensity profile along this line. This profile is compared to the rows of the depthograph to find the best matching position, which is the row of the depthograph that minimizes the distance to the profile. Here the distance we defined is the integral of the squared difference between two pixel intensity profiles. Proceeding this way for each bead in a frame allows us to find their relative positions along z, using the depthograph as a common scale.

In practice, we use this algorithm on the 2 beads pinching the cortex of a cell. In each frame of the film, we determine the center-to-center distances along the 3 axis: dx, dy and dz. Finally, the 3D-distance is:

We estimated the error on dz to be of the order of 50nm and the error on D3 to be 10nm. MORE DETAILS.

## Magnetization, magnetic moment and magnetic force

When using superparamagnetic Dynabeads, it is possible to determine the attractive force a bead applies on another nearby bead as a function of the external magnetic field and the bead positions. First, the bead magnetization M (A/m) as a function of the external field B (T or mT) is known from the literature (5). It was fitted by Julien Heuvingh as an empirical function of this form:

The values of parameters a, b, c, α, β, γ are fixed for all bead types. The value of K is adjusted for each bead type and depends of the density of magnetic particle in the beads. The parameter kcorrMag is adjusted for each new batch of beads purchased (see section on the magnetization measurement).

Then, the total magnetic moment of a bead can be expressed as:

with:

Where V and D are the volume and diameter of the bead. Those are measured once for each batch of beads purchased and treated as constant for all beads from this batch.

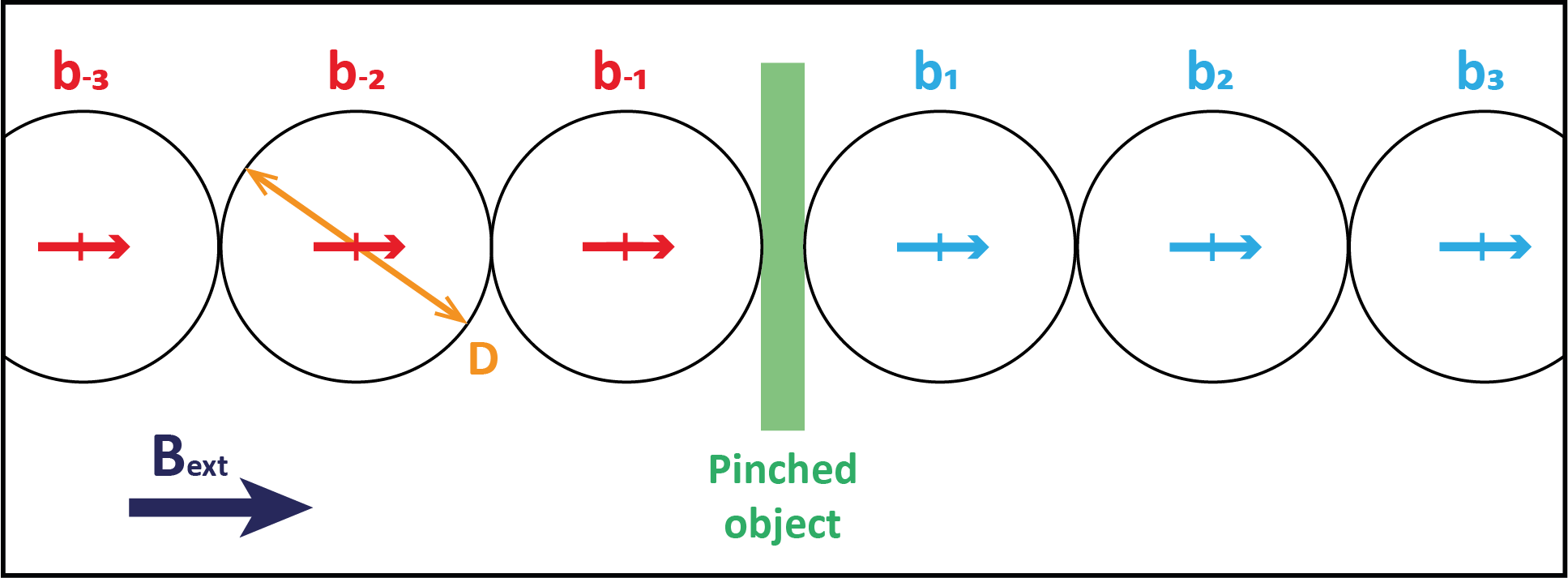
The magnitude of the attractive force exerted by a bead on another is noted F and expressed as such:

Where m1 and m2 are the two beads’ magnetic moments, d is the center-to-center distance, α is the angle between the direction of the magnetic field and the center-to-center direction, and µ0 is the vacuum magnetic permeability (Fig X). The factor expresses that this force is attractive of maximum magnitude when α = 0 (beads aligned with the field), and decreases as α increases, to become repulsive when α > 55°. Note also that F is proportional to d-4, meaning that the attraction decrease quickly as the distance increases. For instance, if F(d = D) = F0 then F(d = 2.D) = F0/16, and F(d = 3.D) = F0/81.

Typical curves for M(B) and F(B) are shown on Fig X. The typical magnetic field range used in our experiments is 1 to 55mT. Note that the attractive force varies significantly in this interval, but grows slower for higher magnetic fields. This is due to the saturation of M(B), typical of paramagnetic materials. This shows that it would be unproductive to use the Magnetic Pincher with much higher fields: the increase in force would be lower and lower.

## Correction of the magnetic force in a chain of beads

When computing the force applied on the cell cortex by beads in the context of the Magnetic Pincher, the situation is usually a bit more complex, because the beads will tend to attract all their neighbors to form chains aligned with the magnetic field direction. Let’s consider the case where an elastic object is pinched by two arbitrary long chains of beads, represented on Fig. X. The applied force is mostly due to the two beads in contact with the object, but the other beads of the chains also contribute, in two ways: increasing the local magnetic field, and attracting beads further away in the other chain. We assumed that a chain can be represented by series of punctual magnetic moments, aligned with the chain direction, and spaced apart by D (the bead diameter).



**Induced magnetic field.** In a chain, each bead is a magnetic dipole which generate its own magnetic field. It adds up with the external field to increase the magnetization of the neighboring beads. The expression of the field generated by a magnetic dipole along its axis is the following:

Hence if a bead has a neighbor distant of one diameter D, the total field is:

The last equality requires neglecting the induced field in the calculation of the neighbor’s magnetization. Based on this formula, we chose to neglect the field induced by beads further than the first neighbors (at a distance 2D, the induced field is already 8 times lower).

In practice, when analyzing an experiment, we wanted to determine the field in the location of the two beads pinching the cortex. We considered only the two following cases:

1. The bead has only one neighbor, which is the other bead pinching the cortex. The correction applied was then:
2. The bead has two neighbors: the other bead pinching the cortex, and a third bead on the other side. Then the correction was computed as:

Where h is the measured cortex thickness.

**Added forces.** When an object is pinched between two chains, the total force applied is not only due to the two beads directly in contact, but also include a contribution from the other beads in the chains. With the notations of Fig. X, the total magnetic force applied on the object results of the attraction of (b1) by (b–1), plus the attraction of (b1) by (b–2), by (b–3), …, plus the attraction of (b2) by (b–1), (b–2), (b–3), etc. Of course, the attractive force between two beads that are very far apart is negligible, since it varies as d–4. Thus we can again disregard beads beyond the first neighbors.

In practice, when analyzing an experiment, we wanted to determine the total pinching force applied on the cortex. We considered only the three following cases:

1. There are only two beads pinching the cortex (b–1 and b1). The force is then simply:
2. One of the two beads pinching the cortex has another neighbor (b-2 or b2).
3. Both of the two beads pinching the cortex has another neighbor (we have b-2, b-1, b1 and b2).

With:

And F–2,1 is computed similarly. In order to simplify the calculations, the angle α was taken to be zero in the expressions of the added forces (F–2,1 and F–1,2). Furthermore, only the magnetic moments of the two main beads m–1 and m1 were corrected with the induced magnetic field. Finally, beads further away in the chains were disregarded.

**Table Y —** **Significance of the corrections.** As an example, here are some numerical values taken from experimental data, corresponding to the situation observed on Fig. X.

|  |  |
| --- | --- |
| **Context (see Fig. X)** | |
| Case / Beads implied | 2 neighbors each / b-2, b-1, b1 and b2 |
| Bead type | Dynabeads M-450 |
| Bext | 5.8 mT |
| h | 977 nm — note that this is an unusually large cortex. |
| F without corrections | 56.7 pN |
| **Correction with induced magnetic field** | |
| Bind | 1.3 mT |
| Btot | 7.1 mT |
| F with corrected field | 75.4 pN |
| **Correction with added forces** | |
| F–2,1 = F–1,2 | 4.7 pN |
| Ftot = F +F–2,1 + F–1,2 | 84.7 pN |

|  |
| --- |
| BOX — Added forces  Let’s consider the case where an elastic object is pinched by two arbitrary long chains of beads, represented on Fig. X. The applied force is mostly due to the two beads in contact with the object, b-1 and b1. But the other beads of the chain also add a contribution. To clearly identify it, let’s do the balance of forces on the chain (b1, b2, b3, …). The chain is attracted by Fmag the magnetic force exerted by the other chain, and this force is balanced by Fel, the repulsive elastic response of the pinched object. Here, Fmag can be expressed as the sum of all attractive forces applied by a bead of the left-hand chain onto a bead of the right-hand chain:  Where Fi,j is the force applied by the bead i on the bead j. However, each of these terms varies as di,j-4, which means that the contribution of the pairs (b-2, b1) and (b-1, b2) is already about 16 times lower than (b-1, b1). For the next neighbors, it goes does down to 81 times lower, etc. Hence, in all of our calculations involving chains of beads, we neglected the contribution of neighbors beyond the first ones: |

# Magnetic Beads Handling

## Bead size measurement

The distribution of diameters of the M-450 Dynabeads has a very low variance, with the exception of rare and obvious outliers, and varies only slightly from one batch to another. Nonetheless, for each vial of beads newly purchased, it is necessary to measure precisely the average diameter, as it is one of the main sources of uncertainties of the technique. This is done using long chains of beads.

Briefly, we coated a 35 mm glass-bottom petri dish with 1 % bovine serum albumin (Merck, Germany). We prepared a solution of the beads of interest in PBS (roughly 200’000 beads/mL) and added 2 mL in the dish. We placed this dish in our imaging set-up (transmitted light, objective 100X NA = 1.4) under a uniform magnitude field of magnitude 5 mT. The beads self-organize to form long chains. We took pictures of chains until we had at least 200 beads in total. Using the bead center detection routine detailed above (BOX), we determined to distance between all pairs of neighbors in the chains. The average was taken as the central tendency for the bead diameter (in a given batch). Measured values can be found below in table Y.

## Bead magnetization measurement

The formula [Eq. Z] captures well the typical magnetization curve of Dynabeads.

However, to account for batch-to-batch variability, we developed a protocol to measure the magnetization of these beads in our lab and adjust the value of accordingly in Eq. Z.

To do so we prepare a small chamber containing a very dilute suspension of beads, which we place in a gradient of magnetic field, using a single electromagnetic coil (Fig. X). Then we film the motion of these beads. In the regime of constant velocity, the force balance projected on the x-axis gives:

The magnetic force experienced by the bead — due to its magnetic moment and the external gradient of field — is balanced by its viscous drag (Fig. X). Being at low Reynolds number, this viscous drag is given by Stokes’ Law : ; where µ is the viscosity of water, R the bead radius and vx the velocity of the bead along the x-axis. On the other hand, once projected, the magnetic force is:

Hence we have the relation:

Using a gauss-meter, we measure the magnetic field & the corresponding gradient produced by the coil according to the current intensity supplied. Then for several values of intensity, we film beads in suspension in water being moved by the magnetic force. As shown on the kymograph on Fig. X, we reach a constant velocity regime and we can measure *vx*. Since R, V and dB/dx are known, we obtain an experimental M(B) curve. By fitting it with Eq. Z, we adjust the parameter and obtain the relation that will characterize this batch of bead magnetization (Fig X). Measured values can be found below in table Y.

Table Y — Measured values for the D and in different batches.

|  |  |  |
| --- | --- | --- |
| **Bead batch** | **D ± std (nm)** |  |
| M450-2020 | 4453 ± | 1.05 |
| M450-2022 | 4503 ± | 1.05 |
| M450-2023 | 4477 ± 18 | 1.023 |
| M450-2025 | 4493 ± 29 | 0.969 |
| M450-Strept | 4506 ± 17 | 1.056 |
| M270-2022 | 2691 ± | 1.05 |

## Bead preparation

Before using M-450 Dynabeads it is necessary to rinse them from the stock solution medium and coat them with a molecule to tune the ability of cells to ingest them. Our way of doing this evolved throughout the project. Originally we were simply incubating the beads with complete medium to let them be coated by serum components. This was inherited from the time when the Pincher was mostly used on Dendritic Cells, which are very prone to ingest objects in their environment.

However it was not very satisfying for 3T3 fibroblasts. During the preparation of an experiment, when we want the beads to be taken in, the rate of ingestion was quite low. Worse: during the experiment itself, when we want the outside-beads to stay out, we observed that bringing the beads close to the cells with the magnetic field led to a very high ingestion rate. In about an hour, we would see many cells completely full of beads, with no beads to be seen in the environment.

This is why we decided to make two different coatings: one intended to increase the ingestion rate by cells, for beads added during the experiment preparation. And the other intended to decrease as much as possible the ingestion, for beads added just before the experiment, which are supposed to remain outside. For the former we used a fibronectin coating and for the latter we found a way to attach PEG chains on the beads. The use of these two distinct populations of beads clearly increased the number of cells properly pinched by beads in our experiments (almost by a factor 2).

**Bead rinsing.** The commercially available M-450 Dynabeads are conserved in distilled water with a concentration of 4x108 beads/mL. Regardless of the coating step we started by rinsing them with the following protocol:

1. Vortex the stock solution vial to re-suspend the beads and pipet 30µL of the solution in an aliquot.
2. Add 1mL of PBS to this aliquot and vortex the mix for 20 seconds.
3. Hold the aliquot vertically above a magnet for 20 seconds. The lower tip of the aliquot should be in contact with the magnet. The beads will sediment rapidly.
4. While holding the aliquot vertically above the magnet, gently remove the supernatant to disturb the bead pellet as little as possible.
5. Repeat steps 2 to 4 twice.

**Coating with complete medium.**

1. In the aliquot containing the rinsed pellet of beads, add 100 µL of complete medium used for the culture of your cells of interest.
2. Vortex the mix for 30 seconds.
3. Before the beads sediment, place the aliquot on a rotating wheel for 3 hours.
4. Conserve the aliquot at 2 to 8°C, with the cap wrapped in Parafilm. If manipulated in sterile conditions, it can last up to one month.

**Coating with fibronectin:** follow the complete medium coating protocol, using 100 µL of fibronectin solution [10 µg/mL fibronectin in NaHCO3 buffer pH 8.3, filtered with a 0.2 µm membrane — the same than the one used for micropatterns] instead of the complete medium.

**Coating with PEG:** identical to complete medium coating protocol, but replacing Dynabeads M-450 Epoxy by M-450 Dynabeads functionalized with streptavidin [included in the CELLection™ Biotin Binder Kit, #11533, Thermo Fisher, USA]; and replacing the complete medium by 100 µL of mPEG(5K)-Biotin solution [#JKA3097, Merck, Germany, 1 mg/mL in HEPES 10 mM, pH 7.4].

In all three cases, the resulting concentration expected in the aliquot is 120’000 beads/µL. Accounting for the loss of a fraction of the beads during the rinsing steps, the actual concentration should be around 100’000 beads/µL. To verify one can dilute the solution 100-fold and count the concentration of beads with a typical cell-counting slide.

## Beads imaging

The 3D tracking of Dynabeads relies on simple bright field microscopy images. However, to obtain a precise tracking, the illumination settings of the microscope need to be tuned, following the guidelines in the BOX below.

|  |
| --- |
| BOX — Microscope settings  Before imaging M-450 Dynabeads, the microscope and camera setting have to be adjusted following these principles:   1. The brightness of the light spot below the beads reaches a maximum a few µm below the equatorial plane (around 3.7 µm on our setup for M-450 Dynabeads). We call the position the ‘plane of maximum intensity’. 2. First, focus on a bead in the plane of maximum intensity. Set the exposure time to 5 ms. 3. Set the Köhler illumination conditions on the microscope, then slightly open the field diaphragm to make the light spot sharper. Verify by moving the focal plane up and down. The light spot spreads a bit as you move away from the maximum intensity plane (Fig. 5C). 4. Adjust the light source power to use a significant fraction of the camera dynamic range. Typically, adjusting the maximum intensity to roughly 40’000 grey levels on a 16-bit camera is ideal. The image should never become saturated throughout the light spot. |

When taking a time-lapse of beads pinching a cortex, the beads might be positioned in different ways with respect to the cell: sometimes perfectly aligned, sometimes a bit diagonally, other times slightly above the other. It happens also frequently that beads get displaced by the cell activity during the time-lapse. As often when doing image processing, the best way to optimize the 3D-tracking is to optimize the image quality during the experiment. To do so we (empirically) developed the following strategy (BOX).

|  |
| --- |
| BOX — Focus strategy  When acquiring a time-lapse, the following points are keys to image the beads to allow a precise 3D tracking:   1. The tracking of the beads in the XY plane will be more precise when the focus is done on the plane where the light spot is the brightest (plane of maximum intensity). 2. The tracking of the beads along the Z-axis will be more precise when the focus is done on a plane located above or slightly below the plane of maximum intensity. This is because the shape of the light spot does not vary much when moving up and down from the plane of maximum intensity, but starts forming specific patterns when one move further below or – even better – above. These patterns facilitate the tracking along Z. 3. As mentioned before, the ideal solution to optimize tracking in both XY and Z is to acquire a small stack of 3 images for each time point. Typically 3 planes 0.5 µm apart in Z and centered on the plane of maximum intensity will allow a very precise 3D localization of the beads. 4. If the conditions of imaging do not allow such Z-stack to be acquired at each time-point, the best plane to localize the bead precisely in XY and Z on a single image is one located slightly above the plane of maximum intensity, where the light spot below the bead starts spreading a little bit: it is the best compromise between points (1) and (2). |

# Magnetic Pincher I – Setup

## Magnetic field generation

|  |
| --- |
| *# This could be placed here or before, in the presentation of the technique in the results section.*  This technique requires the generation of a uniform magnetic field over the experimental chamber. When exposed to an external magnetic field, superparamagnetic beads will become magnetic dipoles, with a magnetic moment that is a function of the applied field. Because the gradient of the external field is negligible, there is no long-range magnetic force and the beads do not drift. Instead, the dipolar force generated by each bead causes them to attract each other. This leads to a self-organization of the beads present in the experimental chamber, forming pairs or chains aligned with the magnetic field (Fig. 5A, 4B). We insist here on the importance of the field uniformity: too strong a gradient of magnetic field would cause superparamagnetic beads to drift toward the higher field regions. |

In this work, we used mainly electromagnetic coils to generate the field. This solution required designing a custom stage to be mounted on a microscope, but has a crucial advantage: a control in real time of the field magnitude. As a consequence of Ampere’s law, an electric current circulating through a coil of conductive wires generate a magnetic field that curls around the coils. A pair of such coils positioned symmetrically along a common axis, with a distance between the coils of the same order of magnitude than the radius of the coils, are called pseudo-Helmoltz coils. If the same currents circulate through both coils in the same direction, a quasi-uniform magnetic field will be generated in the space between the coils. The magnetic field magnitude in the center of this system is proportional to the intensity of the electric current.

In practice, our two coaxial coils (custom made by SBEA Technologies, France) are completed with a mu metal core (750 spires; length: 40 mm; inner diameter: 46 mm; outer diameter: 86 mm, see Fig. 4A) to increase the generated field. They are powered by a bipolar operational power supply amplifier 6A/36V (Kepco, USA) controlled by the computer through a data acquisition module (National Instruments, USA). The maximum field generated is 55 mT (which correspond to the maximum supplied current, 6 A) with a gradient less than 0.1 mT·mm−1 over the sample.

We describe further in the chapter another solution to generate a uniform magnetic field, which is lighter, easier to build in a lab and to mount on any microscope. It is called the Halbach array (8), and consist in a set of permanent magnets arranged in a specific geometry around the sample. Its main downside is to be restricted to constant fields.

## Imaging system

To monitor the actin cortex thickness in time, a pair of beads pinching the cortex must be tracked in 3D. This is done by acquiring a time-lapse movie of the beads illuminated in bright field, with the focus on the light spot below the beads. A high magnification and a high dynamic range are required to produce images where the beads can be tracked precisely.

*# Should this be here or elsewhere?*

When maintaining a constant field, the image acquisition frequency can be low (≈ 1.5 Hz). This allow us to capture a short Z-stack at each time point (3 slices, 0.5 µm apart). Using 3 images at different altitude for a single timepoint increase significantly the precision of the tracking along the z-axis.

Conversely, when filming a compression of the cortex, we used a high acquisition frequency (65 to 100 Hz), which is not compatible with the capture of Z-stacks. We lose a little bit of spatial resolution to increase the temporal resolution. *# End*

Altogether, in addition to the magnetic field generator, the imaging setup includes the following elements:

1. 100X objective, NA = 1.4.
2. Bright-field light source with a field diaphragm and a condenser diaphragm on the light path.
3. SHUTTERS
4. Orca Flash4 camera (Hammamatsu, Japan) with a 16-bits dynamic range.
5. PIFOC focus scanner to control the position of the focal plane along the Z-axis (PIFOC P-721.CDQ, Physik Instrumente, Germany).
6. Image acquisition software which can control both the camera and the Z-axis, and save along with the images a log file containing the precise acquisition time of each image. We developed a Labview software, which we combined with a “DAQ” — a data acquisition module (NI 6343, National Instruments, USA).
7. An environment control chamber to maintain the temperature of the sample to 37°C throughout the experiment, and ideally control the C02 concentration. We use The Box and The Cube (Life Imaging System, Switzerland).

## Inputs & Outputs

**Inputs**. The Labview software purpose is to generate a set-point signal, which is sent to the different elements of the set-up through the data acquisition module (DAQ). This signal contains four components, commanding respectively the camera, the PIFOC Z-displacement, the magnetic field generator and the shutters state. We coded an interface to let the user design an experiment by controlling these four components.

In practice, for constant field experiments, we let the field be constant at 5 mT and we used the PIFOC to capture short Z-stacks every 600 ms. Each Z-stack consists in 3 slices, with a delay of 50 ms and a step of 0.5 µm, ideally centered on the plane of maximum intensity. We captured films of 5 to 10 minutes, sometimes with one image of the cell taken with epifluorescence every minute.

For compression experiments we repeated 5 to 10 times a sequence comprising several phases, represented on Fig. X.

1. A resting phase, with a constant magnetic field, and Z-stack of 3 slices taken every 600 ms (just like in constant field experiments).
2. The pre-compression phase, when the field is lowered progressively to a low point.
3. The compression phase, when the field goes from the low point to a high value, increasing as time squared. The image acquisition frequency is increased to a high value (typically 80 Hz).
4. The relaxation phase when the field is brought back to its resting value.
5. Another resting phase, identical to the first.

The precise values for the different fields are given on table Y. While these two are our standard experimental routines, the software we designed is flexible and allows other types of experiments to be conducted.

**Outputs**. The setup produces two types of raw data. First, films of beads pinching cells, in .tif format, which are used to compute the beads position and eventually the cortex thickness over time. Second, each film comes with a table of numbers, in .txt format, containing the following information for each image:

1. A precise timestamp in millisecond
2. The value of the magnetic field set-point
3. The position of the PIFOC
4. The status of the image in the sequence (resting phase, compression, relaxation, etc.)

In practice, during an experiment, the camera sends a trigger signal to the DAQ module every time an image is captured, and the module acquire in response the numeric data listed above. This ensures a precise correspondence between the images in the film and the associated data in the table.

Table Y — Typical loop structure in compression experiments

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Phase** | **Resting Phase 1** | **Sigmoid down** | **Constant Field** | **Ramp up** | **Ramp down** | **Resting Phase 2** |
| **Duration (s)** | 6 | 2 | 2 | 1.5 | 1.5 | 6 |
| **B(t)** | constant | sigmoid | constant | t² | t² | constant |
| **B initial (mT)** | 5 | 5 | 2 | 2 | 55 | 5 |
| **B final (mT)** | 5 | 2 | 2 | 55 | 5 | 5 |
| **Z-stack** | Yes, 3 slices,  Δz = 0.5 µm | No | No | No | No | Yes, 3 slices,  Δz = 0.5 µm |
| **Delay between frames (ms)** | 50 ms within a stack, 500 ms between | 100 ms | 100 ms | 12 ms | 100 ms | 50 ms within a stack, 500 ms between |

# Magnetic Pincher II – Experimental execution

Overall, the protocol consists in three phases: first, preparing the materials a few days before the experiment (make the patterned chambers and incubate cells with beads to ingest); second, on the day of the experiment, seed the cells in patterned chambers, let them adhere and add external beads (and the drug treatment if required); third, place the chamber in the experimental set-up and run the Labview program to successively film cells with a pinched cortex.

## Cell handling

**Incubation of cells with beads [**2 days before the experiment]

1. Seed approximately 2 × 105 cells in a 25 cm² culture flask with culture medium.
2. Mix 6 µL of the coated M-450 Dynabeads solution in 1 mL of warm culture medium. Vortex and add to the flask. Gently rock the flask to homogenize the distribution of beads.
3. After 2 days, a significant fraction of cells are expected to have ingested at least one bead (10 to 50% according to the bead preparation). Verify that this is the case using a simple phase contrast microscope.

**Preparation of the experimental chamber** [up to one week before the experiment]. Make a micropatterned chambers following the protocol previously detailed. The final step, addition of fibronectin, is done just before the experiment (either the evening before, or in the morning of the experiment day).

**Cell seeding in the experimental chamber** [On the day of the experiment]

1. Prepare imaging medium: around 50 mL culture medium supplemented with 20 mM sterile HEPES buffer (#H0887, Merck, Germany).
2. Detach 3T3 cells from their flask with TrypLE (#12605036, Thermo Fisher, USA) and resuspend them in warm imaging medium. Adjust the quantity of medium to get approximately 1.5× 105 cells/mL.
3. Transfer 2 mL of cell suspension in a micropatterned chamber. Let the cell adhere to the patterns for 20 min in the incubator (37°C 5% CO2).
4. Flush the bottom of the chamber to remove non-adherent cells. To do this, hold a pipet in each hand and inject warm imaging medium on one side as you aspirate on the other side of the chamber. The flux of medium efficiently removes excess cells, leaving only the adherent ones. Check that this is the case and if not, repeat until most floating cells are gone.
5. Let the chamber for 2 hours in the incubator (37°C 5% CO2) so that the cells adhesion mature.

**Outer bead addition (without drug treatment).** In an aliquot, prepare a mix of 0.5 mL of warm imaging medium and 2.5 µL of the coated beads solution. Vortex the mix and add to experimental chamber.

**Outer bead addition (with drug treatment)**

1. Adjust the volume of medium in the experimental chamber to 1 mL
2. In an aliquot prepare a mix of 1 mL of warm imaging medium, 2.5 µL of the coated beads solution and the chosen drug at twice the target concentration
3. Vortex the aliquot and add to the chamber 30 minutes before starting imaging

## Magnetic Pincher imaging

After placing the sample onto the microscope stage, the first step is to acquire Z-stacks of single beads. They will be used to generate a depthograph, as described previously. It is preferable to perform this step in the experimental dish containing the cells in the absence of a magnetic field: the formation of chains of beads would hinder the acquisition of clean Z-stacks.

**Z-scan of beads (depthograph)**

1. Set the microscope light power and the field aperture as explained in BOX. These setting should not change throughout the whole imaging session.
2. Position one or several beads in the field of view. These beads should be clearly separated from each other, perfectly still, and their image not affected by any object in the chamber or on the optical path. Position the focus in the equatorial plane of the beads (see BOX).
3. Acquire a Z-stack of these beads: 401 steps every 20 nm, for a total course of 8 µm. The piezo element must be used to ensure precision of the displacement along Z.
4. Repeat steps 2 and 3 until at least 8 Z-stacks of beads have been successfully acquired.

Then, the magnetic field can be applied to trigger the beads self-organization in the whole chamber, and many pinching event should occur simultaneously. One can start looking for a cell whose cortex is pinched by beads, and use the Labview program to film the bead displacement under a constant field, or perform series of compressions.

**Magnetic Pincher on live cells**

1. Switch on the electromagnetic coils’ power supply. The resting field magnitude we used was 5 mT, and was kept constant across experiments to ensure results are comparable.
2. Position a cell whose cortex is pinched by a pair of beads in the field of view. In addition to the guidelines defined in BOX, the following criteria must be met:

* These beads should be as much as possible aligned along the magnetic field lines and in the same plane.
* The light spot below them should not be hindered by extra or intra-cellular objects.
* The pair of beads can be part of a longer chain that extend into, or out of the cell, or even both. However, beads pinching the cortex must have a maximum of 2 nearest-neighbors (one of them being automatically the other bead of the pair) that are well aligned with the line of magnetic field. Otherwise the magnetic field in the region of the beads will not be properly defined.

1. Acquire a time-lapse of these beads. It consists either in an observation under constant magnetic field, or in a series of compressions.

* *Constant field experiment.* 5 to 10 minutes, one Z-stack of images every 600 ms, for a total of 200 to 1000 time-points. Each Z-stack consists in 3 slices, with a step of 0.5 µm, ideally centered on the plane of maximum intensity and with a delay of 50 ms between each step of the Z-stack.
* *Compression experiment*. Series of 5 to 10 sequences comprising a resting phase, a compression phase where the field is brought to its maximum value, a relaxation phase where the field is brought back to its resting value, and another resting phase. See the ***Inputs & Outputs*** section for a detailed description.

1. Repeat steps 2 and 3 to acquire a dataset representative of your cell population. A given chamber should not be images for longer than 2 hours. Typically 10 to 20 cells should be acquired in each chamber, depending on the duration of the acquired time-lapse movies.

# Magnetic Pincher III – Image analysis

This section details the image analysis workflow used to extract the cortical thickness as a function of time from the acquired time-lapse images. It requires two different software: ImageJ (in our case Fiji (9)) and Python. Overall, given raw data from one experiment chamber, we started by generating a Depthograph using the acquired Z-stack of single beads. Then a simple tracking algorithm is applied to the Magnetic Pincher films, to compute the trajectories of the beads in 3D. Like most tracking algorithms, ours proceed in two steps: (i) Objects segmentation and (ii) Frame-to-frame matching. Step (i) is done semi-automatically with Fiji while step (ii) has been automatized in a Python function.

The Python code can be found on Github (Constant field experiment only: <https://github.com/jvermeil-biophys/CortExplore_MIMB>; Constant field and compressions: <https://github.com/jvermeil-biophys/CortExplore_OpenMecha>). The main libraries required to to run the code are the following: os, time, numpy, pandas, scipy, scikit-image (10), matplotlib and pyautogui.

|  |
| --- |
| BOX — Bead center detection in Fiji  This detection method is routinely used across many of the image analysis workflows presented in this chapter. While simple, it allows a detection of the center of a bead in the XY plane with a very high precision. It consists in segmenting of the light spot below a bead, then computing its center of mass, using the pixel value as weight (see Fig. 7).   1. In the Fiji software, open an image or a movie containing beads (Fig. 7A). 2. In “Set Measurement” select “Area”, “Standard deviation”, “Center of Mass”, “Stack Position”. 3. Use “Image/Adjust/Threshold” to manually segment the light spots under the beads (Fig. 7B, 6C). Enable the “Stack histogram” option. If the file analyzed is a movie, the same threshold value is used to segment the beads in all the frames of the movie. The regions segmented should cover each light spot, be roughly circular and have a diameter around 1 to 2 µm. Leave the “Threshold” dialog box open with the thresholded regions visible on the image. 4. Use the “Image Particle” tool to measure the properties of each region (Fig. 7D), with the following options enabled: “Display results”, “Exclude on edges”, “Include holes”, “Clear results”. Adjust the area and circularity criterion so that the only objects analyzed are the segmented light spots. Select “Show: Outlines” to visually check that all the light spots were analyzed. 5. Save the Results table (Fig. 7E) in .txt format. |

## Generate a Depthograph from the bead Z-stacks

In order to make a reference Depthograph, process bead Z-stacks acquired for a given experiment to compute a typical YZ profile of the beads.

**Detection of the beads center**, in Fiji.

1. In the Fiji software, open a Z-stack of one or several beads. For each bead crop a small rectangular region of interest around the bead and save the resulting Z-stack in .tif format.
2. Open one of these single-bead Z-stack.
3. Use the method detailed in BOX to detect the position of the center of the bead on most frames of the Z-stack. Not managing to detect the light spot on the first and the last frames is normal. The light spot should be successfully segmented typically from frame 100 to frame 300 over a total of 401 in the stack, without any misdetection within this range. Check visually with the “Outlines” that it is the case.
4. Save the Results table in .txt format in the same folder as the corresponding .tif single-bead Z-stack.

**Computation of the Depthograph**, in Python.

This task is performed using a custom Python script (see the Github repositories for a more precise documentation). Here is the outline of the algorithm we used.

1. Open a .tif Z-Stack of a bead. Using the previously computed Results file, on each frame, translate the image so that the center of mass of the bead is exactly in the center of the image. This is done using skimage transform.warp() function with a bi-linear interpolation.
2. On each frame, take the vertical intensity profile of the bead. This can be done by averaging the 5 central vertical lines of the frame. This intensity profile is a 1D array of pixel value.
3. Concatenate all these arrays along a second dimension to obtain a 2D array of intensity profiles. The first dimension is Y the second is Z. This constitutes a Depthograph for a given bead.
4. Repeat the steps 1 to 3 for each stack of beads corresponding to an experiment. Average all the Depthographs, by using the plane of maximum intensity to align them. The resulting 2D array is the average YZ profile of beads imaged to produce the Depthograph of a given experiment.

## Segment the beads with ImageJ

As a first step to track the beads positions in the Magnetic Pincher time-lapse, segment the light spots of the beads of interest in all the frames in ImageJ / Fiji and determine the position of their center.

1. In the Fiji software, open a Magnetic Pincher time-lapse. If necessary convert it to .tif format.
2. If the images are organized as Z-T hyperstacks, flatten to obtain a 1-dimensionnal stack where images are ordered in the following way: (t1, z1), (t1, z2), (t1, z3), (t2, z1), (t2, z2), (t2, z3), …, (tN, z1), (tN, z2), (tN, z3); where t1 … tN are all the successive time-points and z1, z2, z3 are the lower, middle and upper Z-planes, in that order. To do so, use the function: “Image/Hyperstack/Hyperstack to Stack”.
3. Use the method detailed in BOX to detect the position of the center of each bead of interest in the time-lapse. Check visually with the “Outlines” plot that the light spots corresponding to the beads of interest were analyzed in nearly all the frames. Save the Results table in .txt format.
4. Repeat steps 1 to 3 for each of the acquired Magnetic Pincher time-lapse.

## Track the beads in 3D with a custom-made Python algorithm

The second step of the tracking is performed using another custom Python script. Here are the main steps of the algorithm.

1. For each Magnetic Pincher time-lapses file, the first frame is displayed and the user selects the two beads pinching the cortex.
2. The program matches the positions of the beads frame by frame to build their trajectories in the XY-plane. If in a frame, one bead is not found or its displacement is deemed too high, the program displays this frame to ask the user to confirm the bead position (if possible) or the impossibility to track the bead on this frame.
3. Once trajectories are built, for each of the beads of interest the user fills in whether it is in or out of the cell, and whether it has 1 or 2 neighbors (necessary to compute the pinching force).
4. The displacement of the beads along the Z-axis are computed by comparing for each frame the vertical profile of the beads of interest to the Depthograph generated for this experiment.
5. From the XY and Z positions of the beads, the center-to-center distance in 3D is computed for each timepoint.
6. The force applied by the beads on the cortex for each time-point, using the calculation and the corrections detailed previously.
7. Finally, the program saves the result as a .csv file.

The resulting “Time-series” file is a table containing the evolution with time (column “T”) of different quantities: the beads center-to-center distances along each axes (columns “dx”, “dy”, “dz”), in the XY-plane (column “D2”) and in 3 dimensions (column “D3”). It finally contains the magnetic field (column “B”) and the force (column “F”). To compute the time-resolved thickness of the cortex, subtract the beads average diameter to the 3D distance.

|  |
| --- |
| BOX — Try the code!  \*Instructions to find the code on GitHub and try it on example data!\* |

# Magnetic Pincher with Halbach Arrays

The term “Halbach array” refers to an arrangement of permanent multipole magnets. These arrays can have very diverse properties and application depending on their geometry. Here we use a set of dipolar magnets arranged in a circle (for more details see (11) and specifications in Table 1). It generates a uniform magnetic field inside the circle, and a negligible field outside. The magnets have to be identical in size and magnetization, and arranged so that their direction vector rotates twice faster than their position vector on the circle (Fig. 2A).

In such geometry, the field generated in the center of the Halbach array has the following magnitude:

[Eq. Z]

Where N is the number of magnets used in the array, µ0 is the vacuum magnetic permeability, R is the radius of the circle joining the center of magnets, and m is the magnitude of the magnetic moment of the magnets.

Another useful relationship in Halbach array design is the following: for a given magnet of volume V that contains a uniform field of magnetization M, the resulting magnetic moment m is simply:

[Eq. Z]

As a reference the neodymium N42, of which the cubic magnets we use are made, has a magnetization of MN42 ≈ 1.01 x 106 A/m. Their magnetization is simply deduced from this value and their geometry.

A slightly more complex application of this concept is the nested Halbach array (Fig. 3B). The idea is to use two concentric Halbach array that generate the same field B0 and can be rotated independently. This is possible using larger magnets for the outer array, since as stated by Eq. Z, the generated field magnitude decreases rapidly when the radius of the array increases; this decrease is compensated by increasing the volume of the magnets so they possess a larger magnetic moment (Eq. Z). Therefore, this system can generate uniform magnetic field of any magnitude between ≈ 0 mT (when the two arrays’ fields are in opposite direction) and 2.B0 (when they are in the same direction).

We detail here the specifications of a nested array that we have optimized for this protocol and which is easy to mount on many microscopes, given that it has the outer dimension of a 6 well plate (Table 1, Fig. 3C). The body of the device, meaning the rectangular support and the two rings bearing the magnets, have been 3D-printed (Printer: Fortus 250mc, Stratasys, USA; Material: ABS X-TREME, iSQUARED, Switzerland). The neodymium cubic magnets are commercially available (Supermagnete, Germany). This instance of the nested Halbach array is designed to generate a maximum field of 2.B0 = 8.6 mT.

**Table 1** – Example of nested Halbach array design – Technical specifications

|  |  |
| --- | --- |
| **Support** | |
| Length x Width x Thickness | 127 x 85 x 3.5 mm |
| **Inner array** | |
| Inner / Central / Outer radius | 21 / 24.75 / 28 mm |
| Height | 5 mm |
| Magnet side length | 3 mm |
| Number, type of magnets | 16, N42 Neodymium |
| **Outer Array** | |
| Inner / Central / Outer radius | 28 / 33 / 38 mm |
| Height | 5 mm |
| Magnet side length | 4 mm |
| Number / type of magnets | 16 / N42 Neodymium |
| **Magnetic Properties** | |
| Maximum magnetic field magnitude  (arrays in the same direction) | Ideally: 8.6 mT  Experimentally: 8.3 mT |
| Minimum magnetic field magnitude  (arrays in opposite directions) | Ideally: 0 mT  Experimentally: 0.2 mT |
| Gradient over the central 1 cm region | < 0.11 mT.mm-1 |
|  |  |

**Table 2** - Comparison of the two magnetic field generation solution

|  |  |  |
| --- | --- | --- |
|  | Halbach array | Coils |
| Fabrication | * The body can be 3D-printed * Magnets are available at low cost | * Need of a custom manufacturing |
| Size | Can be designed with the size of a 6-wells plate or a 10 cm petri dish. | Each coil is 40 x 86 mm  (length x outer diameter) |
| Mounting on a microscope | Simple, given the flexible design options. | Require a ≈ 140 x 95 mm rectangular hole in the microscope stage. |
| Generated field | With a simple array: one fixed field, from 1 to 90 mT.  With a nested array: tunable field, from 0 to 30 mT. The adjustments cannot be done live during an experiment. | Field adjustable in live during the experiment by tuning the intensity of the current supplied to the coils. The field can go from 0 to 60 mT, but high magnitudes cannot be maintained too long, due to the Joule effect heating the coils. |

# Magnetic Pincher with Nano-Indenter

We developed a set-up to measure on the same cell the mechanics in a local point of the cortex and at the scale of the whole cell, successively. The idea was to combine our Magnetic Pincher with something as similar as possible to an AFM with a wedged cantilever.

To do so, we chose the Chiaro nanoindenter (Optics11 Life, the Netherland) which offers a decisive advantage compared to most AFM-like apparatus: the probe is placed at the tip of an arm. This enabled us to use this device in association with the electromagnetic coils, which occupy a substantial space on our microscope. We had to design a new custom stage to accommodate for both systems (sig Fig. X).

Overall, the functioning principles of the Chiaro nanoindenter differs only slightly from an AFM: the idea is still to indent the sample with a sphere placed at the tip of a soft cantilever. As one lower the base of the cantilever to press the sphere onto the sample, the resulting deflection of the cantilever is measured. This way one can compute the applied force (cantilever stiffness multiplied by its deflection) corresponding to the indentation depth (displacement of the cantilever base minus cantilever deflection). The originality of the Chiaro system resides in the deflection measurement, illustrated on Fig. X. Briefly, an infra-red laser is sent in an optical fiber placed vertical above the cantilever tip. First the light reaches the fiber/medium interface and is partially reflected; then the transmitted part reaches the medium/cantilever interface and is reflected. The two reflected signals are collected by the optical fiber and they produce Fabry-Perot interferences. Finally the cantilever’s deflection can be deduced from this interference pattern. Indeed the fringes’ distribution depends on the difference in distance covered by the two reflected signals, which varies linearly with the deflection. For more details, see (12).

Beside the probe integrating the cantilever and the optical fibers mounted on a glass ferule, the other key elements are the OP1550 interferometer, which generates the laser and measures the interference pattern (Optics11 Life, the Netherland); and the piezoelectric element which controls finely the vertical motion of the probe.

## Modifications of the setup

In order to accommodate the Chiaro nanoindenter, we had to adapt the geometry of the microscope stage and the position of the coils. Those also had to be placed a bit further from each other, resulting in a lower maximum magnetic field in compressions experiments. The microscope incubator also had to be adapted to fit the rod supporting the Chiaro head. Otherwise, the setup was left functionally unchanged by the addition of the nanoindenter.

## Execution

Our goal was to conduct successive measurements on the same cell with the Magnetic Pincher and the Chiaro nanoindenter. Overall, the preparation of such experiment was almost identical to our standard compression experiment (see above), with a few notable changes:

* The Phenol Red in our usual imaging medium absorbs the infrared laser used by the Chiaro, so we used a transparent imaging medium instead. Fluorobrite (#A1896702, Thermo Fisher) with 1% v/v GlutaMax Supplement (#35050038, Thermo Fisher), 10% v/v FBS (#S1810-500, Biowest), 1% v/v PS (#15070063, Thermo Fisher), and 20 mM sterile HEPES (#H0887, Merck). This medium was in the micropatterned chambers as soon as the cells were seeded.
* LifeAct-EGFP cells

Briefly, series of combined measurements were conducted in the following way: after placing the micropatterned chamber on the microscope stage, the probe of the Chiaro nanoindenter was prewetted with a few drops of medium and immersed in the chamber. The probe was lowered close to the bottom of the chamber and centered so as the sphere at the tip of the cantilever would be in the center of the camera field of view. The nanoindenter was then calibrated (see below). Then, the probe was moved-away (see below). Quickly, a few single beads Z-stacks were acquired for depthograph generation. The magnetic field was applied, and a suitable cell was located and indented with the Magnetic Pincher (series of 5 compression, see Magnetic Pincher II). Then the nanoindenter probe was brought back in the field of view, and centered above the cell previously pinched. Following the routine detailed below, the cell was indented 2 or 3 times. The probe was once again moved away and a new cell suitable for the Magnetic Pincher was located to continue the measurements. In total, 4 to 7 cells were measured in a dish, so as the total duration of the experiment does not exceed 1h30. Before removing the chamber, the probe of the nanoindenter was fully brought up, and a drop of medium was added to keep the probe wet while the next chamber was brought to the microscope. The probe did not systematically required a new calibration for each new chamber.

**Chiaro calibration.** The calibration of the probe coupled with the OP1550 interferometer was largely automatized by the Chiaro software. It requires the probe to be immersed in the measurement medium and consists in two steps:

* Optical calibration: the OP1550 interferometer automatically tunes its parameters (laser wavelength, photodiode gain and offset) so that the interference pattern is a good measure of the cantilever deflection.
* Geometrical factor calibration: the probe is lowered so that the tip of the cantilever makes contact with a stiff substrate (the glass bottom of our chamber is suitable). Then the device indent the substrate twice, so that it can automatically compute its “geometrical factor”. This number is a correction applied on each probe, and captures the fact that the deflection measurement point is not perfectly above the tip of the cantilever. It also depends of the medium optical index.

**“Move away - Move in” routine.** As the Chiaro probe is immersed in the chamber, a meniscus forms around it. The curvature of the air-medium interface greatly affects the transmitted light path and the aspect of bright field images captured. A simple solution to this problem is to use a fixed routine to move the probe away — to do bright field imaging — and bring it back in — to perform indentations. In our case we raised the probe by 2 mm and translated it by 5 mm (in the XY-plane) to remove the meniscus effects; and performed the same operation backward to bring the probe back in the field of view. Thanks to the precision of the device, the probe returned almost exactly back to its former position.

**Indentation routine.** The main objective of this routine was of course to obtain a force-indentation curve, but it also include the measure of other important geometrical parameters. After bringing back the probe in the field of view, it was centered on the cell in the XY-plane and positioned a few micrometers above. We switched to epifluorescence microscopy to visualize LifeAct-EGFP in the cell and captured an image of the cell basal plane, so we can measure the radius of the cell-substrate contact. Then the cell was indented with the following sequence, designed to measure the cell height on top of the force-response:

1. The probe is centered above the cell
2. Move for 30 µm in XY
3. Automated “Find surface” step and move up for 20 µm
4. Fast indentation-retraction against the glass surface: total displacement 25 µm, speed 10 µm/s.
5. Move back onto the cell (30 µm in XY)
6. Slow indentation of the cell: total displacement 12 µm, variable speed (0.5 to 4 µm/s, typically 1 µm/s).
7. Hold for 20 seconds
8. Retraction: displacement and speed identical to indentation.

This convoluted routine is what we found optimal to be able to measure precisely and robustly the height of the cell as the indentation is performed. Indeed, step 3 allow us to position the probe roughly 20 µm above the substrate. Step 4 make use of the piezo-controlled z-axis to determine with precision the height above the substrate. By comparing the contact point found in this first indentation and the one found in step 6 when indenting the cell, we can determine the cell height.

While the cell is indented, it is possible to observe it in bright field because the central region of the sphere is transparent. This is an easy way to confirm that the indentation effectively happened and that the probe was properly centered. However, the outlines of the cell are hardly visible. To observe the variation in projected area during the indentation, we filmed the cell in epifluorescence to visualize LifeAct-EGFP. The reflected light path is not hindered by the sphere & cantilever, and let us observe clearly the outline of the cell.

This sequence was repeated 2 to 3 times for each cell, depending on the quality of centering of the probe with respect of the cell. Simply, the first indentation was observed in transmitted light to confirm the centering accuracy. If it was deemed insufficient, adjustments were made and another indentation with transmitted light was done to verify. The last indentation was made together with epifluorescence imaging, to film the cell deformation.

**Note on the total duration of the experiment**. The probe of the Chiaro accesses the cells from the top of the chamber. Naturally, the lid needs to be removed to do so. In a 37°C atmosphere, it resulted in a higher evaporation rate: around 0.3 mL per hour. This is problematic as it causes the osmolarity of the medium to slowly increase with time. To limit this effect, several solutions could be envisioned but we lacked time to implement them, so we decided to simply limit the maximum duration with the lid removed to 1h30.

## Analysis with the Hertz model

A semi-infinite, isotropic, linearly elastic material indented by a spherical probe of radius Rp has the following force response F:

with Y the Young modulus, ν the Poisson ratio and δ the indentation depth. In this work we will commonly use an “effective Hertz contact modulus” which is our fitting parameter, and expressed as:

This model is obviously far from physically correct when applied to the indentation of a cell, which respects almost none of the hypotheses. However, this model fits quite well F- δ curves obtained in experimental cell indentations, and it is still very common in AFM-based studies. For these reasons we used it as a first way to characterize the cell mechanics and compare cells.

To fit our experimental F- δ curves, the model was first fitted on the whole curve, including the approach phase. More precisely we fitted:

Where z is the position of the sphere apex of the sphere along a descending z-axis and Z0 is the point of contact (so as δ = z – Z0). Z0 and KH were the fitted parameters. With this first estimation of the point of contact, we rejected curves for which the approach phase was less than 1 µm long and where the maximum indentation was less than 2 µm. Then we repeated the fit over a smaller interval spanning from Z0 – 1 to Z0 + 3 (µm).

## Analysis with the elastic envelope model

The cytoplasm, when seen as a viscoelastic gel, has an exceedingly low storage modulus (13). The cortex is seen as the main contributor to the whole cell mechanics. In this logic, experiments of single-cell compression between parallel plates (wedged AFM) where interpreted in terms of cortex mechanics (14,15). To do so, the cell was modeled as a thin viscoelastic envelope — the cortex — containing a constant volume of liquid — the cytoplasm. The force response during the compression is caused by the stretching of the envelope, shear and bending are neglected. These models differ on the constitutive equation they use to account for the envelop mechanics. However, they agree on the geometrical computation of the cortex stretching, by assuming cells envelope behave as Delaunay surfaces, where minimizing the free energy equals finding solutions of constant total curvature. Overall, these models constitute a flexible approach to whole-cell mechanics modelling, where one can compute the cell geometry as the compression happens, and in a second step pick a mechanical constitutive equation to fit the force response.

Our experiments differed in two ways from those analyzed in (14,15): our cells are adhering on fibronectin patterns (non-zero contact angle with the substrate) and instead of parallel plate we indented the cells with large spherical beads (around 3 times larger than the cells). However, this case was also described in (15); the calculations I develop below are therefore mostly identical to theirs, but were slightly adapted to account for geometries they did not consider.

**Solving the geometry of an indented cell, first case**. Because the cortex is a thin envelope (100 to 800 nm), bending and shearing are neglected and the free energy can be computed from its stretching alone:

Where T is the envelope tension, KA is area compressibility modulus and α is the areal strain, defined as:

The geometry minimizing the envelope free energy is therefore the solution with minimal surface area. Since we are considering cells adhered on disc-shaped patterns, indented from above by a sphere, the system is symmetrical by rotation around the z-axis. In such case, the solutions of constant volume have been shown to be the surfaces of constant total curvature and are called Delaunay surfaces. This can be demonstrated using calculus of variations, and was done synthetically in (15).

Let us first consider the situation depicted on Fig. X, where the contact angle Φ is below π/2. Applying the Laplace law to the cell envelope gives:

Where Cθ and Cϕ are the curvatures along the polar and the azimuthal directions, respectively. In a cylindrical coordinate system, both curvatures can be expressed as functions of with γ the angle of the normal vector with the z-axis:

Hence we get:

Integrating this relation gives a solution of the form:

With two boundary conditions in r1 and R1:

With this, A0 and B0 can be expressed as functions of the cell geometry:

|  |  |  |  |
| --- | --- | --- | --- |
| BOX – Spherical cap  For a spherical cap as represented if Fig. XB, we used the following expressions for the height, surface area and volume:   |  |  |  | | --- | --- | --- | |  |  |  |   Note that these three parameters depends only of Rp and r1. |

Knowing the values of A0 and B0 is enough to compute the position of the cell envelope, its surface area and its volume. Indeed, it is simple to see on Fig. XC that:

Using this formula, we obtain the following expressions:

If we note z1 = z(r1) we can also express the indentation δ as:

Where H0 is the height of the cell before the compression, and hcap the height of the spherical cap as defined in BOX.

Of all the geometrical parameters used here, we know H0 and R1, which are measured during the experiment, and we also know Rp, which is given by the probe manufacturer. The two unknown parameters are r1, the radius of the cell-bead contact area, and Φ, the cell angle of contact. Determining these two parameters allows us to fully determine the cell contour geometry. To find them, we can solve numerically the following system of two non-linear equations:

Where δset is the applied indentation — which we can set to any value within the range that correspond to the experimental compression [0, δmax] — and V0 is the original volume of the cell, which can be expressed from R1 and H0 (assuming a constant curvature the initial shape of the cell is a fraction of a sphere).

To summarize, the shape of the cell can be computed for any value of δset; we simply have to define the complete expressions of δ and V as functions of r1 and Φ, and we solve the above system numerically for these two unknown, using the function fsolve of the library scipy.optimize in Python.

**Solving the geometry of an indented cell, second case.** This reasoning, however, is not general enough to cover all the cases we encounter in our experiments. Indeed, as shown on Fig. XA, it happens that the cell is quite larger than the pattern size, and its initial contact angle will be higher than π/2. Alternatively, the compression can increase the contact angle until it goes above this critical value. In such case, we need to define 2 sub-regions of the cell, because z(r) is no longer a properly defined function over the whole contour. On Fig. XB is the geometrical parametrization we used to solve this case: region 1 encompass the part of the contour where z is above z0, and r varies from r1 to R0. Region 2 is the part where z is below z0 and r goes from R1 to R0. We could apply the same reasoning as before to each region:

Table Y — Equations describing the cell envelope geometry when Φ > π/2

|  |  |  |
| --- | --- | --- |
|  | Region 1 | Region 2 |
| Differential equation |  |  |
| Expression of |  |  |
| Boundary conditions |  |  |
| Coefficients of |  |  |
| Contour position |  |  |
| Surface area |  |  |
| Volume |  |  |

Using these expressions we can finally compute the indentation, as well as the total surface area & volume for the cell envelope:

We have now three unknown parameters: r1 and Φ, like before, but also R0, the maximum radius of the cell. Thus our system needs to include a third equation, which intuitively needs to link the two regions. Indeed, one of our fundamental hypotheses is the fact tension is conserved across the whole envelope. Total curvature in region 1 and region 2 should be equal. This can be written as:

Which leads to:

This is the equation needed to close our system:

Once again, we solve the above system numerically for r1, Φ and R0, using the function fsolve of the library scipy.optimize in Python.

**Arbitrate between the two cases.** To resolve all compressions with the same program, we devised a simple algorithm:

1. If the cell already has a contact angle above 90°, it always falls in the second case, with the two regions.
2. If it does not, we compute whether the range of δ applied in the experiment is enough to make the cell transition from the one region case to the two regions case.
   1. If it does not, it always falls in the first case, with only one region.
   2. If it does, we compute a δ limit, which is the delta for which Φ = π/2. Then we split the range of values for δset. For δ < δset we used the 1 region solver, for δ > δset we used the 2 regions one.

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**In region 1:**

with two boundary conditions in r1 and R0:

and A1 and B1 can be expressed as:

**Similarly in region 2:**

with two boundary conditions in R1 and R0:

and A2 and B2 can be expressed as:

1. The project on membrane-to-cortex attachment is presented in annex of this thesis. The other two collaborations were mostly driven by my team mate Anumita Jawahar, and will not be further discussed here. [↑](#footnote-ref-1)
2. https://www.nature.com/articles/s41598-019-48370-3 [↑](#footnote-ref-2)